TAUTOMERIC AND ANOMERIC SPECIFICITY OF ALLOSTERIC ACTIVATION OF YEAST PYRUVATE KINASE BY D-FRUCTOSE 1,6-BISPHOSPHATE AND ITS RELEVANCE IN D-GLUCOSE CATABOLISM

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1. Introduction

In aqueous solution D-fructose 1,6-bisphosphate (FBP)† exists as equilibrium mixture of 20 ± 4% α-D-fructofuranose 1,6-bisphosphate, 80 ± 10% β-D-fructofuranose 1,6-bisphosphate, and less than 1.5% of the open chain [1–4]. The two anomeric forms are spontaneously interconvertible via the open chain tautomer. The first order rate constant for the ring opening of the α-D-fructofuranose 1,6-bisphosphate is 0.55 sec^{-1} at pH 7.6 and 25°C [4]. The terms 'tautomeric' and 'anomeric' are used here according to the definitions given in a previous report [5].

D-fructose 1,6-bisphosphate takes part in four reactions of D-glucose metabolism: it is the product of D-fructose-6-phosphate kinase, the substrate of D-fructosebisphosphate aldolase and D-fructosebisphosphatase, as well as the activator of allosteric pyruvate kinase [6]. The tautomeric and anomeric specificities of the first three enzymes have been studied by several research groups [4–5, 7–10]. Fructose-6-phosphate kinase [5,7–9] and fructosebisphosphate aldolase [4] are specific for the β-fructofuranose form of their substrates, although the latter enzyme can cleave the open chain form of FBP also. On the other hand fructosebisphosphatase has a preference for the α-fructofuranose form [10].

In this paper we wish to report the results of an investigation on the tautomeric and anomeric specificity of the allosteric FBP site of yeast pyruvate kinase. Our approach is to use structurally locked analogues of each of the three forms of FBP. Because of structural characteristics, these compounds cannot tautomerize or anomerize in solution. 2,5-anhydro-D-glucitol 1,6-bisphosphate is used as an analogue of the α-form, 2,5-anhydro-D-mannitol 1,6-bisphosphate as an analogue of the β-form and hexanediol 1,6-bisphosphate as an analogue of the open chain form. Our data show that the analogue of the β-form is an excellent allosteric activator of yeast pyruvate kinase, whereas the analogue of the α-form and the open chain form exhibit only a limited stimulatory effect on the enzyme. It is concluded that β-D-fructofuranose 1,6-bisphosphate is the true allosteric activator of yeast

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†Enzymes: Fructosebisphosphatase, D-fructose 1,6-bisphosphate 1-phosphohydrolase (EC 3.1.3.11). Fructosebisphosphate aldolase, D-fructose 1,6-bisphosphate D-glyceraldehyde-3-phosphate lyase (EC 4.1.2.13). Fructose 6-phosphate kinase, ATP:D-fructose-6-phosphate 1-phosphotransferase (EC 2.7.1.11). Lactate dehydrogenase, L-lactate: NAD^+ oxidoreductase (EC 1.1.1.27). Pyruvate kinase, ATP: pyruvate phosphotransferase (EC 2.7.1.40).

pyruvate kinase. The relevance of the anomeric specificity of fructose 6-phosphate kinase, fructosebisphosphate aldolase, fructosebisphosphatase and the allosteric site of pyruvate kinase in glucose metabolism is also discussed here below.

2. Materials and methods

All chemicals used were p.a. grade and purchased from E. Merck AG., Darmstadt, if not otherwise stated. D-fructose 1,6-bisphosphate tetracyclohexylammonium salt, ADP, NADH, phosphoenol pyruvate, and lactate dehydrogenase (from rabbit muscle) were bought from Boehringer Mannheim GmbH. The compounds 2,5-anhydro-D-glucitol 1,6-bisphosphate, 2,5-anhydro-D-mannitol 1,6-bisphosphate and hexanediol 1,6-bisphosphate were synthesized from their respective polyols according to the procedure described elsewhere [11]. However, we have found that this procedure yields appreciable quantities of isomeric bisphosphates and some trisphosphates as by-products. These had to be separated by added purification steps as follows. The intermediate 2,5-anhydro-D-mannitol 1,6-bis-(diphenylphosphate) was purified by dry column chromatography (Woelm silica gel) and the 2,5-anhydro-D-glucitol 1,6-bisphosphate by DEAE-25 (Pharmacia) column chromatography. The final products were isolated as their cyclohexylammonium salts. Each compound was characterized and its state of high purity was ascertained by nmr spectroscopy, paper chromatography, elemental analysis and optical rotation.

Yeast pyruvate kinase was purified as described in [12] with minor modifications. Methods for the determination of substrate concentration as well as enzyme activities were as described in [13]. Most of the ammonium sulfate was removed from the suspension of lactate dehydrogenase and pyruvate kinase by centrifugation and the residual enzymes dissolved in a buffer having the following composition: 50 mM dimethylglutaric acid–NaOH, pH 7.0, containing 100 mM KCl and 30 mM $MgSO_4$. The kinetic experiments with yeast pyruvate kinase were performed in this buffer in a final vol. of 1 ml with 0.3 mM NADH, 5 mM ADP, phosphoenol pyruvate (varied concentrations), FBP or one of its analogues (varied concentrations) and 50 units/ml of lactate dehydrogenase. An experiment without effector was performed as a control. All reactants were dissolved in the above described buffer. The reaction was initiated by addition of an appropriate amount of pyruvate kinase and followed at 366 nm using an Eppendorf photometer connected to a recorder. The temperature was 25°C.

3. Results

Initial velocity studies of the pyruvate kinase reaction were carried out at different concentrations of phosphoenol pyruvate in the absence of activator or in the presence of 5 mM FBP (equilibrium concentration of its isomeric forms) or 5 mM of one of the three analogues mentioned above.

The saturation curves obtained in these experiments with respect to phosphoenol pyruvate are depicted in fig.1. The interaction coefficient n_H, V_{max} (maximal velocity) and $K_{0.5}$ (substrate concentration giving v = half V_{max}) were computed from the data of fig.1 by the program of Wieker et al. [14] and are summarized in the table. It is clearly seen from Fig.1 and the table that the analogue of the β-furanose form of FBP is an excellent allosteric activator of yeast

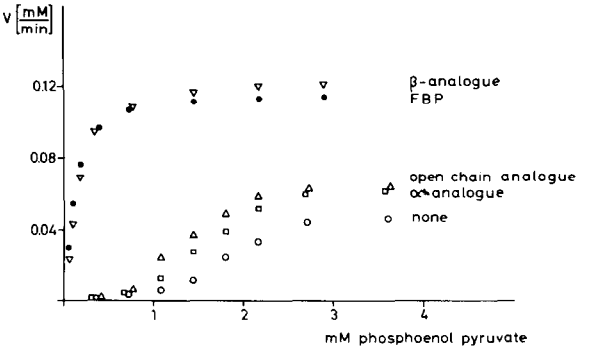

Fig.1. Initial velocities (mM × min^{-1} = μmoles × ml^{-1} × min^{-1}) of the pyruvate kinase reaction at different concentrations of phosphoenol pyruvate and saturating concentration of Mg-ADP (5 mM) in the absence of any effector (○, none), in the presence of 5 mM of the equilibrium mixture of FBP (●, FBP), as well as in the presence of one of the analogues: 2,5-anhydro-D-glucitol 1,6-bisphosphate analogue of α-furanose FBP (□, α-analogue), 2,5-anhydro-D-mannitol 1,6-bisphosphate analogue of β-furanose FBP (▽, β-analogue), and hexanediol 1,6-bisphosphate analogue of open chain FBP (△, open chain analogue); 25°C and pH 7.0.

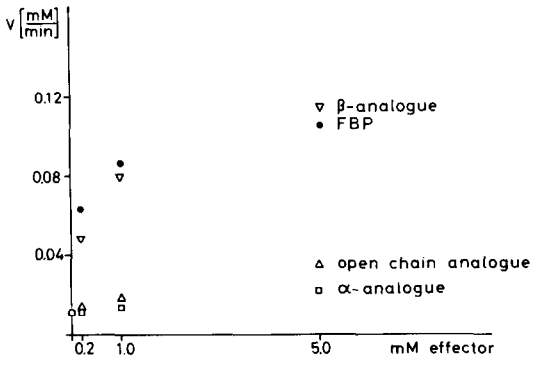

Fig.2. Initial velocities of the pyruvate kinase reaction at 1.44 mM phosphoenol pyruvate and saturating concentration of Mg-ADP (5 mM) in the absence of any effector (open circle on the ordinate) and in the presence of different concentrations (0.2 mM, 1 mM and 5 mM) of the effectors: equilibrium mixture of FBP (•, FBP), analogue of α-furanose FBP (□, α-analogue), analogue of β-furanose FBP (▽, β-analogue), and analogue of open chain FBP (△, open chain analogue); 25°C and pH 7.0.

pyruvate kinase. It yielded kinetic parameters comparable with those obtained with FBP as activator. On the other hand the analogues of the α-furanose form and the open chain form of FBP brought about only marginal activation. Three series of experiments, performed at slightly different concentrations (activities) of yeast pyruvate kinase, yielded the same results.

In the experiments described above, the effectors were tested at 5 mM final concentration. We performed similar experiments using FBP or one of its analogues at 0.2 mM, 1 mM and 5 mM final concentration in the presence of 5 mM Mg-ADP and 1.44 mM phosphoenol pyruvate. The results obtained in these experiments are given in fig.2. At all three concentrations of the effectors, yeast pyruvate kinase is strongly activated by the β-analogue and only marginally affected by the α-analogue or the open chain one.

4. Discussion

If one uses structural analogues of biochemical compounds in order to elucidate the specificity of enzymes, one has to ask the question: how closely related are the analogues to the physiological metabolite? In the experiments reported here we used 2,5-anhydro-D-glucitol 1,6 bisphosphate and 2,5-anhydro-D-mannitol 1,6-bisphosphate as analogues of the α-furanose and β-furanose forms of FBP, respectively. These two analogues have identical structure and configuration as the corresponding anomers of FBP with exception of C-2 where the hydroxyl group is replaced by a H atom. This permutation prevents the tautomerization and anomerization of these compounds in solution. Hexanediol 1,6-bisphosphate was used as an analogue of the open chain form of FBP. Although this analogue differs from FBP at carbon atoms number 2, 3, 4 and 5, it still resembles the latter in having an open chain of six carbon atoms and two phosphate groups at C-1 and C-6.

The data we presented here show that only the analogue of the β-furanose form of FBP is a potent activator of allosteric yeast pyruvate kinase. The analogues of the α-furanose form and the open chain form of FBP exhibited only marginal activating effect. In addition, it should be mentioned, that in earlier experiments it was shown that the two phosphoryl residues of FBP are required for maximum allosteric activation [15].

In the following scheme the anomeric specificities of fructose 6-phosphate kinase, fructosebisphosphate aldolase and allosteric pyruvate kinase are summarized. All three enzymes show specificity for the β-anomer of FBP. Thus, fructose 6-phosphate kinase specifically

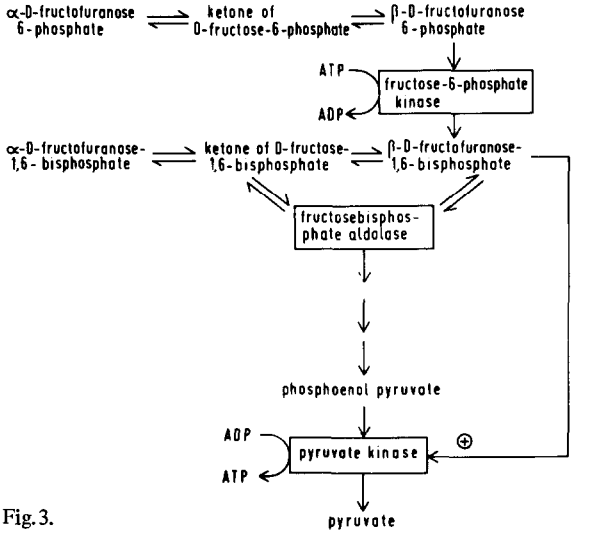

Fig.3.

catalyzes the phosphorylation of β-D-fructofuranose 6-phosphate [5,7–9] yielding β-D-fructofuranose 1,6-bisphosphate. The latter can be accepted as substrate by fructosebisphosphate aldolase [4] and the β-anomer of FBP functions also as an allosteric activator of pyruvate kinase.

It should be noted that the enzymes investigated were prepared from different biological sources. Both fructose 6-phosphate kinase and fructosebisphosphate aldolase were from rabbit muscle whereas pyruvate kinase was from yeast. A priori, one cannot conclude that analogous enzymes from other biological sources exhibit the same tautomeric specificity. However, in the cases of hexokinase, glucose 6-phosphate dehydrogenase and glucosephosphate isomerase, the tautomeric and anomeric specificities of analogous enzymes from various biological sources were found to be identical [16].

The identical specificity for one form of FBP of the three reactions of the glycolytic pathway considered here seems to be a manifestation of an optimal fit. Since the product of the fructose 6-phosphate kinase reaction (β-FBP) can be accepted as substrate by fructosebisphosphate aldolase there is no spontaneous or enzyme-catalyzed tautomerization or anomerization of FBP intercalated between these two reactions. The coupling via β-FBP of the two regulatory enzymes of glycolysis, fructose 6-phosphate kinase and pyruvate kinase, could be relevant during transient states in which the intracellular concentration of FBP might rise a hundred fold, i.e., from 0.02 mM to 2 mM [17]. The increase in concentration of total FBP is initially an increase in β-FBP which can immediately cause an activation of pyruvate kinase. Thus, a rapid increase in the glycolytic flux can be brought about through the activation of pyruvate kinase at low concentrations of phosphoenol pyruvate. This minimizes the flux of phosphoenol pyruvate into other pathways. Moreover, the coupling of several reactions via the specificity for one isomeric form of a sugar substrate would be necessary if the enzymes are organized in a multi-enzyme complex in which the product of one reaction is directly transferred to the next enzyme in the reaction chain. It should be added here that there is no direct evidence for a multienzyme complex structure of the glycolytic pathway at the present time, however, the matter has been subjected to wide discussions [18].

Table 1

Kinetic parameters of yeast pyruvate kinase in the absence and presence of its allosteric activator, fructose 1,6-bisphosphate, or one of the analogues of its isomeric forms.

Effector (5 mM)	Hill coefficient n_H	V_{max} (mM/min)	$K_{0.5}$ (mM)
None	3.6	0.051	1.9
Hexanediol 1,6-bisphosphate (open chain analogue)	3.5	0.066	1.3
2,5-anhydro-D-glucitol 1,6-bisphosphate (α-furanose analogue)	3.6	0.065	1.6
2,5-anhydro-D-mannitol 1,6-bisphosphate (β-furanose analogue)	1.2	0.124	0.15
D-fructose 1,6-bisphosphate (equilibrium mixture of isomeric forms)	1.2	0.116	0.10

The kinetic parameters n_H (interaction coefficient), V_{max} (maximal velocity), and $K_{0.5}$ (substrate concentration giving $v = V_{max}/2$) were computed from the data of fig.1 by means of the program of Wieker et al. [14].

Although not shown in the scheme, it is interesting to note that in gluconeogenesis, the α-anomer of FBP is the preferred substrate of fructosebisphosphatase [10]. This is in contrast to the reactions of the glycolytic pathway which are specific for the β-anomer of FBP as mentioned above. It is tempting to speculate that the separation of the pathways for catabolism and anabolism of D-glucose through the selection of different anomeric specificity in the forward and the reverse directions has a significance in minimizing the waste of energy (ATP) in the cycle of fructose 6-phosphate kinase and fructosebisphosphatase [19,20].

After the completion of this paper, a report by Schray et al. [21] came to our attention. It confirmed our results on the anomeric specificity of fructosebisphosphate aldolase from rabbit muscle [4]. However, the authors suggested that the enzyme from yeast also cleaves the α-furanose form of FBP to a minor extent, a result not contradicting our arguments.

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